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Significance and Impact of the Study: This tool clearly improves previously published methods of specific real-time quantitative PCR for *Phytophthora infestans* by allowing the intraspecific detection and quantification of Avr3a/avr3a genotypes. This reliable and sensitive assay has enabled to assess zoospore production as a fitness proxy and thus offers new opportunities for future researches on *P. infestans*/host quantitative interactions.

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Abstract

Molecular tools that allow intraspecific quantification and discrimination of pathogen isolates are useful to assess fitness of competitors during mixed infections. However, methods that were developed for quantifying *Phytophthora infestans* are only specific at the species level. Here, we reported a TaqMan-based real-time PCR assay allowing, according to the specificity of the used probes, an accurate quantification of different proportions of two genetically distinct clones of *P. infestans* in mixed fractions. Indeed, in addition to a primer specific to *P. infestans*, two primers and two TaqMan® probes that target single-nucleotide polymorphisms located in the Avr3a/avr3a virulence gene sequence were designed. The reliability of the method was tested on serially diluted fractions containing plasmid DNA with either the Avr3a or the avr3a sequences at concentrations ranging from 10^2 to 10^8 copies per μl. Based on its specificity, sensitivity and repeatability, the proposed assay allowed a quantification of the targeted DNA sequence in fractions with a Avr3a/avr3a ratio in the range 1/99 to 99/1. The reliability of the test was also checked for counting zoospores. Applications for future research in *P. infestans*/host quantitative interactions were also discussed.

Introduction

Individual fitness is a common measure found in studies attempting to assess and compare the relative success of genotypes in a population of individuals belonging to a particular species (Day and Otto 2001). As intraspecific competition within a host occurs in many host–pathogen systems (Read and Taylor 2001), studying the competitive ability of different genotypes is a key issue for numerous research programs linked, for example, to the evolution of virulence of pathogens (Gower and Webster 2005; de Roode *et al.* 2005b; Bell *et al.* 2006; Choisy and de Roode 2010) or the emergence of new variants (Kadish and Cohen 1988; Rolland *et al.* 2010). Understanding the dynamics of pathogen populations requires knowledge of both the identity and the frequency of the different genotypes infecting the hosts. Thus, fitness investigations at the intraspecies level are only possible within systems in which each individual offspring can be easily assigned to parent or families. Most spore-producing parasites, in which there are no morphological differences between individuals and/or between their offspring,

do not constitute easy biological models for such investigations. Moreover, for these models, it remains difficult to catch, tag or distinguish offspring and linked them to the identity of their parents. Indeed, during competition within a single host, it is impossible to identify the origin of the spores without using markers specific for each individual genotype present in the mixture. These specific genetic markers of individuals can be detected either at the genomic level by molecular tools based on polymorphisms (e.g., microsatellite markers; Montarry *et al.* 2010a) or at the macromolecular level (e.g., use of monoclonal antibodies; Lacroix *et al.* 2010). Recent advances in next-generation sequencing technologies (Shendure and Ji 2008) provided new alternative methods to detect/describe specific genotypes in mixtures using, for example, barcodes (Smith *et al.* 2010) or primer ID (Jabara *et al.* 2011). Nevertheless, these methods cannot be easily (and routinely) applied for accurate quantification of closely related individuals in mixed infected materials.

Phytophthora infestans (the causal agent of potato and tomato late blight) is a hemibiotrophic oomycete causing severe yield and quality losses on crops (Robertson 1991). Its life cycle begins with the penetration of host tissue by germ tubes from sporangia (or zoospores differentiated within the sporangia), followed by mycelial growth within host tissue and formation of new sporangia resulting from clonal (asexual) reproduction, which differentiate uninucleate zoospores (Judelson and Blanco 2005). Thus, the fitness of *P. infestans* can be assessed by quantifying sporangia or zoospores. However, this approach does not allow the quantification of individual fitness during mixed infections. Indeed, all existing molecular tools developed for quantification of *P.* have a detection resolution at the species level (Bohm *et al.* 1999; Atallah and Stevenson 2006; Llorente *et al.* 2010; Lees *et al.* 2012). Although intraspecific discrimination is possible for *P. infestans* using genetic profiles from population genetic tools (Montarry *et al.* 2010a), quantification would remain a problem.

In this manuscript, we report a real-time PCR method based on TaqMan® probe technology that makes it possible to accurately quantify in *P. infestans* mixtures the relative proportions of different genotypes of this plant pathogen. The presented method was developed using intraspecific variations of the gene coding for the Avr3a effector protein because of its well-known polymorphism (Armstrong *et al.* 2005) and because it is involved in rapid potato resistance breakdown (Turkensteen 1993). Avr3a is one of the effectors *P. infestans* secretes within the potato (*Solanum tuberosum*) host tissue to evade recognition by host cells and to suppress or manipulate host defences (Birch *et al.* 2006). Avr3a interacts with the product of the R3a gene (Bos *et al.* 2006, 2009) present at the R3 resistance locus of potato (Huang *et al.* 2004). The outcome of this interaction depends on *P. infestans* genotype, virulent (avr3a) or avirulent (Avr3a), that leads to either infection or not, respectively. Because (i) real-time PCR technology makes it possible to detect and quantify specific genotypes (e.g. Williams *et al.* 1998; Cheesman *et al.* 2003; Dufour *et al.* 2011) and (ii) the developed assay is based on genetic differences between alleles of the Avr3a gene, the proposed quantitative diagnostic tool can be efficiently used to screen virulent and avirulent natural *P. infestans* populations.

Results and discussion

Avr3a- and avr3a-specific detection/quantification

Sequence alignment of the Avr3a gene from the BP3, OEK, BEK, BP6, P46 and P8 *P. infestans* isolates revealed five single-nucleotide polymorphisms (SNPs) among which two (located at positions 241 and 312 in the Avr3a sequence, position according to Armstrong *et al.* 2005) were used as targets in the intraspecific primers and probes design procedure (Fig. 1). These SNPs correspond to changes in amino acid of the resulting protein. Thus, this suggests their possible involvement in the avirulent/virulent phenotype (Armstrong *et al.* 2005; Bos *et al.* 2006, 2009). The BP3 and OEK isolates exhibited the avirulent (Avr3a) genotype, whereas the four other isolates were associated with the virulent (avr3a) genotype. BP3 and BP6 are homozygous at the Avr3a locus. These two *P. infestans* isolates were respectively selected as Avr3a and avr3a reference isolates for the procedure used to develop the real-time PCR assays presented in this work.

When the appropriate primer pair and probe (F-R^{Avr3a}–$Probe^{Avr3a}$ or F-R^{avr3a}–$Probe^{avr3a}$) were used with standards corresponding serially diluted fractions (Avr3a or avr3a, respectively) containing from 10^2 to 10^8 copies per μl, the increase in fluorescence signal observed during the PCR allowed the calculation of standard curves associated with the two developed real-time PCR assays (Fig. 2). The slopes of the produced curves (−3·81 and −3·76 for Avr3a and avr3a quantification assays, respectively) and the determination coefficient ($R^2 = 0{\cdot}99$ for the two assays) indicated that these two real-time PCR assays produce accurate quantifications (in the range 10^2–10^8 DNA copies per tested sample) of samples corresponding to fractions containing one of the targeted sequences (Avr3a or avr3a). The four replicates of the whole experiment (i.e. the entire qPCR run) were associated to slope and efficiency results (see framed graph at the upper right corner in Fig. 2) that validate the

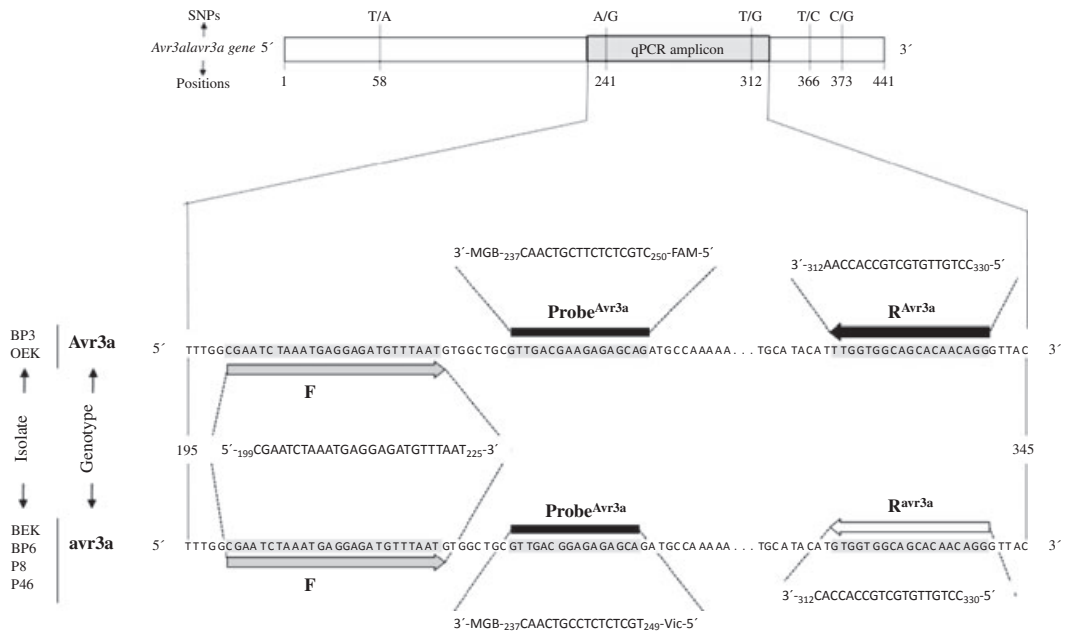

Figure 1 Schematic representation of the single-nucleotide polymorphisms (SNPs) identified along the Avr3a gene from the sequences of six *Phytophthora infestans* isolates (lower part) and specific sequences that were targeted in real-time quantitative PCR assay (upper part). Binding sites for forward unspecific primer (F), specific reverse primers (R^{Avr3a} and R^{avr3a}) and specific TaqMan® probes ($Probe^{Avr3a}$ and $Probe^{avr3a}$) are presented. Their sequences and 5′ and 3′ modifications of probes are detailed.

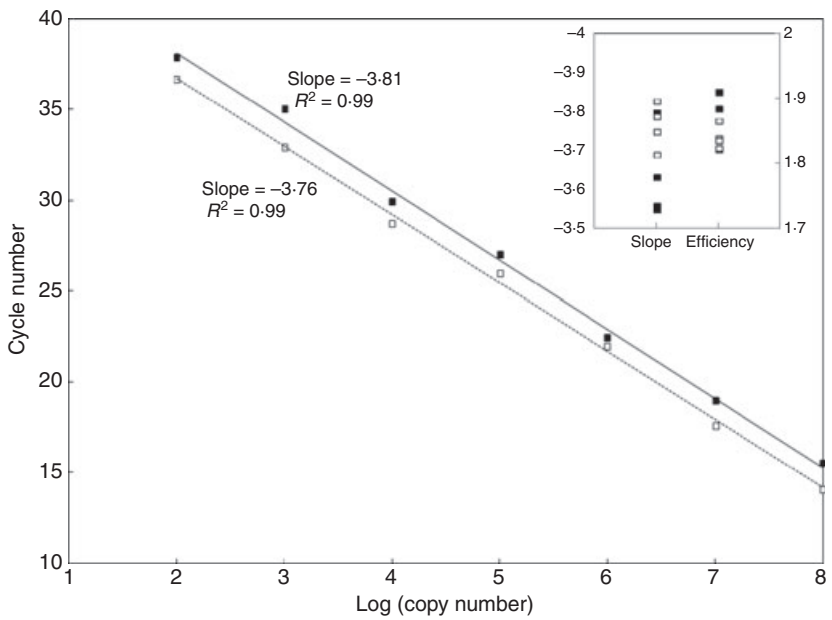

Figure 2 Standard curves for the Avr3a- and avr3a-specific real-time PCR assays developed for *Phytophthora infestans*. Standard curves are illustrated in plain and dotted lines from Avr3a (dark squares) and avr3a (open squares) DNA quantifications, respectively. Values of slope and efficiency for each of the four replicates per specific real-time PCR assay are presented at the upper right corner of the graph.

repeatability of the assays. Moreover, no fluorescence signal was detected during the 45 cycles of the real-time PCR when F-R^{avr3a}–$Probe^{avr3a}$ primer/probe was used to detect/quantify the Avr3a DNA sequences or when F-R^{Avr3a}–$Probe^{Avr3a}$ primer/probe was used to detect/quantify avr3a DNA sequence.

Reliability in mixed DNA fractions

The reliability of the assay was tested using the targeted sequence (Avr3a or avr3a) mixed with the nontargeted one. This procedure was performed with the two DNA sequences (Avr3a and avr3a) at concentrations in a range from 10^2 to 10^8 copies per reaction. Thus, 49 mixtures (Table 1) were twice quantified, once using the F-R^{Avr3a}–$Probe^{Avr3a}$ primers and probe combination, and once using the F-R^{avr3a}–$Probe^{avr3a}$ primers and probe combination. Both detection and quantification of the Avr3a sequence failed at the lowest concentration (i.e. 10^2 and 10^3 copies/reaction, Table 1, NC in standard typo) when the competitor (i.e. avr3a) was at least 10^4 times more concentrated (i.e. 10^7 and 10^8 copies per μl). This phenomenon occurred also for the quantification procedure that targets the avr3a sequence as noticed in the data results illustrated in Table 1 (NC in bold). However, for most quantification data (84/98) associated to the 49 tested artificial mixtures, the Avr3a- and avr3a-specific quantitative assays provided a suitable quantification of the targeted sequence.

According to the acceptable −2/+2 magnitude in the quantification results produced under our experimental condition by the real-time PCR assays, the sequence-dependent specificity of this new assay can be defined using ratios (calculated DNA target quantity (Avr3a or avr3a)/theoretical DNA target quantity present in samples (Avr3a or avr3a)) ranging between 0·5 and 2 as proposed by Balme-Sinibaldi *et al.* (2006). When competitors (Avr3a/avr3a) are present at a ratio within a range 1/99–99/1, the calculated/theoretical ratio values associated with these samples were between 0·5 and 2 except for seven Avr3a/avr3a mixtures (Table 1, underlined data). Nevertheless, under our experimental conditions, the calculated/theoretical ratio values, obtained whatever the tested Avr3a/avr3a ratios, were not below 0·82 or above 2·35.

Between-test variation (reproducibility)

Based on the acceptable −2/+2 magnitude in quantification results (see above), the three other repetitions of the whole qPCR run for both targets (i.e. Avr3a and avr3a) gave expected accurate results. Detection of the target when the competitor was present up to a 100-fold higher concentration is an acceptable range for fitness measures. For each theoretical copy number of target, the mean quantification of four replicates of pure Avr3a, pure avr3a and Avr3a/avr3a quantifications associated with ratios corresponding to 50/50, 10/90, 90/10, 1/99 and 99/1 were compared. Because the design of the experiment corresponds to a crossed table of Avr3a/avr3a concentrations ranging from 10^2 to 10^8 copy number, the proposed

Table 1 Quantification of the Avr3a and avr3a gene copy number of *Phytophthora infestans* in different mixtures containing known theoretical quantities of both target (Avr3a or avr3a) and competitor (avr3a or Avr3a, respectively). For each target quantification, appropriate real-time quantitative PCR assay was used and results were analysed with the LightCycler 480 software (v 1.5.0)

avr3a copy number	Avr3a copy number											
	1·0E+08	1·0E+07		1·0E+06		1·0E+05		1·0E+04		1·0E+03		1·0E+02	
1·0E+08	1·0E+08*	**9·9E+07**	1·5E+07	**8·3E+07**	1·3E+06	**7·3E+07**	9·2E+04	**7·5E+07**	1·1E+04	**7·5E+07**	NC	**7·4E+07**	NC	**7·7E+07**
1·0E+07	9·1E+07	**1·1E+07**	1·2E+07	**1·2E+07**	1·2E+06	**1·1E+07**	9·6E+04	**1·1E+07**	1·4E+04	**1·0E+07**	NC	**1·1E+07**	NC	**1·2E+07**
1·0E+06	9·8E+07	**1·1E+06**	1·4E+07	**1·2E+06**	1·4E+06	**1·2E+06**	1·3E+05	**1·3E+06**	2·0E+04	**1·3E+06**	8·0E+02	**1·4E+06**	NC	**1·4E+06**
1·0E+05	9·2E+07	**3·8E+04**	1·4E+07	**1·3E+05**	1·5E+06	**1·3E+05**	1·2E+05	**1·3E+05**	2·2E+04	**1·4E+05**	8·9E+02	**1·4E+05**	1·7E+02	**1·4E+05**
1·0E+04	1·0E+08	**NC**	1·5E+07	**NC**	1·5E+06	**1·4E+04**	1·3E+05	**1·4E+04**	2·1E+04	**1·6E+04**	1·2E+03	**1·4E+04**	1·4E+02	**1·4E+04**
1·0E+03	1·1E+08	**NC**	1·4E+07	**NC**	1·5E+06	**NC**	1·3E+05	**1·7E+03**	1·9E+04	**1·4E+03**	1·1E+03	**1·5E+03**	8·2E+01	**1·6E+03**
1·0E+02	1·1E+08	**NC**	1·5E+07	**NC**	1·6E+06	**NC**	1·3E+05	**NC**	1·8E+04	**1·6E+02**	1·0E+03	**1·6E+02**	1·4E+02	**2·4E+02**

NC, not calculated.

*Normal font and bold font numbers correspond to Avr3a and avr3a quantifications, respectively. Underlined numbers correspond to quantification results associated with fractions with a calculated DNA target quantity/theoretical DNA target quantity ratio below 0·5 or above 2·0.

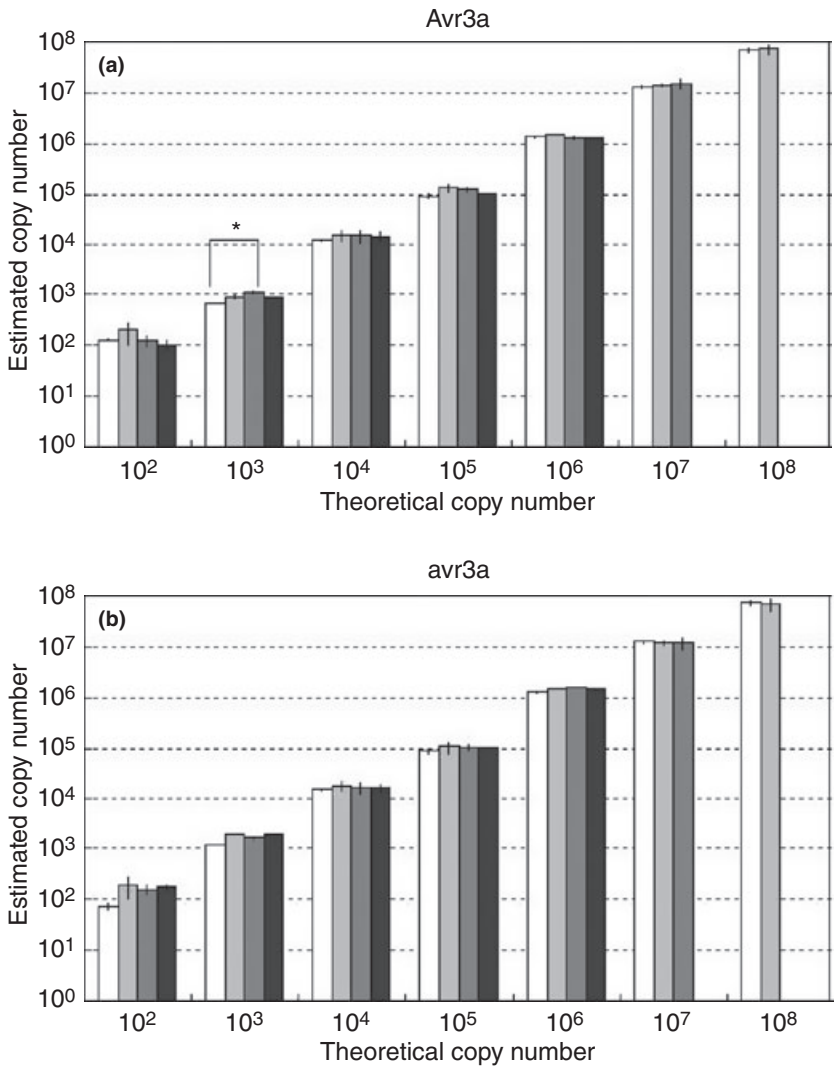

Figure 3 Mean of Avr3a (a) and avr3a (b) targeted DNA copy numbers of *Phytophthora infestans* calculated for the four repetitions of the real-time PCR assay. Pure DNA (white) and mixed fractions corresponding to 50/50 (light grey), 10/90 (dark grey) and 1/99 (black) Avr3a/avr3a ratios are represented. A unique significant difference (*, $P = 0{\cdot}004$) was found between the two means when multiple comparisons were performed between fractions within a theoretical copy number value.

ratios could not be tested at the edge of the Avr3a/avr3a mixture matrix. Indeed, testing the 1/99 ratios was not possible for the two highest concentrations of targets (i.e. 10^7 and 10^8), and testing the 10/90 ratio was not possible for the upper concentration of targets (i.e. 10^8) because mixtures containing 10^9 and 10^{10} copy number of the competitor were not available. The analysis of data produced with the 50 tested mixtures showed an accurate and similar ($P > 0{\cdot}05$) quantification of the targets as denoted by pairwise comparisons performed for each theoretical copy number of the targets (Fig. 3). However, a significant difference between the pure fraction and ratio 10/90 for the theoretical 10^3 copy number of the Avr3a target ($P = 0{\cdot}004$) was found. Despite this significant difference between means, both quantification belonged to the $-2/+2$ magnitude around the 10^3 theoretical value ($6{\cdot}74 \times 10^2$ and $1{\cdot}1 \times 10^3$ for pure and 10/90 mixed fractions, respectively).

Extending the reliability of the test and counting zoospores

Analysis of the DNA fraction from the 12 *P. infestans* isolates tested in this work using the real-time PCR assays allowed the identification of 10 homozygous genotypes [one (BP3 *P. infestans* isolate) Avr3a/Avr3a and nine (BEK, BP6, P13, P43, P46, PON05, PON10, PP1 and Z0 *P. infestans* isolates) avr3a/avr3a] and two heterozygous

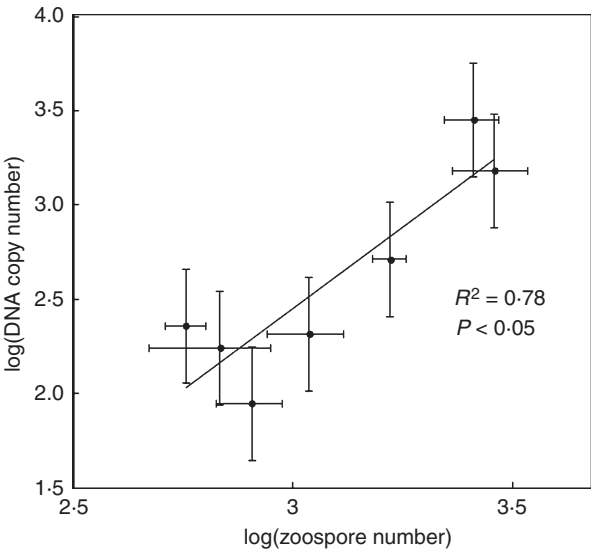

Figure 4 Dot plot of zoospore DNA quantification versus zoospore counting for seven avr3a/avr3a homozygous isolates of *Phytophthora infestans*. Horizontal bars represent standard errors of the mean for eight replicates of optical quantifications, and vertical bars represent the −2/+2 magnitude of acceptable error for real-time PCR quantification. The correlation was significant (R^2 = 0·78, P < 0·05, slope = 1·74).

genotypes Avr3a/avr3a (OEK and P8 *P. infestans* isolates). These results, confirmed by the sequence data of the Avr3a gene, highlight the specificity and efficiency of the proposed real-time PCR assays (Avr3a or avr3a specific) for the genotyping of *P. infestans* isolates.

Zoospore DNA quantification obtained from a subsample of seven randomly chosen isolates among the ten homozygous ones was correlated with zoospore counting obtained from classical microscopy observation. As illustrated in Fig. 4, the number of zoospores estimated by visual counting and the number of DNA copies obtained by real-time PCR assay are correlated (P < 0·05, R^2 = 0·78, slope = 1·73) making it possible the replacement of classical quantification procedures for zoospores counting by the developed molecular quantification procedure. As we targeted specifically a unique copy of the Avr3a gene and as zoospores are uninucleate diploid structures, the slope of the curve presented in Fig. 4 is expected to be equal to two (just be aware that polyploidy could occur in very particular cases as in Judelson and Yang 1998). Because DNA extractions always results in DNA loss, the number of zoospores determined by the molecular method is slightly underestimated. However, zoospore quantification is generally used as a relative rather than an absolute measure of reproductive fitness (for example, zoospores produced in multiple relatively to single infections). This underestimation, which is slightly the same for all samples, does not represent a problem in the assessment of reproductive fitness. Our method thus constitutes a rapid and reliable proxy of zoospore production to assess and compare fitness of different *P. infestans* isolates under different biological conditions.

Epidemiological and ecological applications

The utility of real-time quantitative PCR methods to detect and study phytopathogenic species is obvious (Schena *et al.* 2004). However, among a hundred articles citing the work of Schena *et al.* (2004), only one (Balme-Sinibaldi *et al.* 2006) reported the development of a real-time PCR assay allowing the quantification during competition at the intraspecific level (PVY^O and PVY^N isolates of the potato virus Y). To our knowledge, all others methods consisted in the detection and quantification of pathogens at the intragenus (or even upper) level. The intraspecific approach we developed has not been previously reported for the pathogenic oomycete *Phytophthora infestans*. Moreover, the objective of this work was also to develop a method to easily quantify zoospores from *P. infestans* isolates, which provided a useful tool to deal with epidemiological, ecological and evolutionary questions.

As multiple infections of hosts by different isolates of the same pathogen often occur in natural conditions (Read and Taylor 2001), this parameter has to be included in epidemiological, ecological and evolutionary studies using host–pathogen models. Moreover, this within-host pathogen diversity has been proved to have direct consequences on the host–pathogen interaction outcome (de Roode *et al.* 2005a; Jager and Schjorring 2006; Ben-Ami *et al.* 2008) and to affect pathogen evolution (Nowak and May 1994; van Baalen and Sabelis 1995; May and Nowak 1995). The understanding of life history and evolution of host–pathogen interactions could thus be achieved only at the light of studies dealing with multiple infections. The possibility offered by the intraspecific quantification of *P. infestans* isolates allows the exploration of new questions on the ecology, evolution and epidemiology of the pathogen *P. infestans*. Such real-time PCR assays allow to further explore competitive ability of *P. infestans* isolates and the impact of mixed infections on the reproduction strategy of this pathogen as it was recently published by Clément *et al.* (2012). Furthermore, the developed molecular tool was also validated on DNA extracted from oospores (i.e. structures resulting from sexual reproduction of two compatible *P. infestans* isolates). As oospores are thick-walled resting organs, they can survive for extended periods in the soil in the absence of the host and can serve as primary soil-borne inoculums for at least 2–3 years (Andersson *et al.* 1998) and cause early infections during following growing seasons

(Lehtinen and Hannukkala 2004; Hannukkala *et al.* 2007; Widmark *et al.* 2007). Due to their significant impact on the long-term epidemiology of *P. infestans*, attention must be paid in the understanding of the ecological and environmental conditions leading to their formation. As *P. infestans* is a heterothallic species, sexual reproduction, and thus oospore formation, is possible only when two compatible genotypes (i.e. known as A1 and A2 mating types) come into contact. Dual infection is therefore an obligate stage before sexual reproduction occurs. This could consequently reduce the investment of both genotypes in their asexual stage, which supply the current (i.e. short term) epidemic. By allowing the detection of two different genotypes within a mixture, our molecular tool opens thus new opportunities for future researches on *P. infestans*, such as testing the impact of multiple infections on the differential investment of *P. infestans* in sexual vs. asexual reproduction. Consequences of multiples infections constitute one of the current main challenges for the scientific community working on *P. infestans* and more generally for the scientific community working on the evolutionary ecology of host–parasite interactions.

Avr3a is one of the effectors the pathogen secretes within the potato (*Solanum tuberosum*) (Birch *et al.* 2009). The Avr3a gene product interacts with a resistance gene product of the host and results either in a defence response of the plant leading to an absence of infection (i.e. the hypersensitive response HR) or in an absence of response leading to an infection (Armstrong *et al.* 2005). The interaction outcome depends on the alleles the pathogen possesses (Armstrong *et al.* 2005; Bos *et al.* 2009). Thus, the method presented in this article will be very useful for screening *P. infestans* isolates for their genotype at the Avr3a locus. Indeed, it provides a faster method than the usual time-consuming biotests in which isolates have to be inoculated on the potato host containing the R3 resistance to determine the outcome of their interaction (Andrivon 1994). Based on this proposed real-time PCR detailed method, others similar tools targeting different Avr gene (or others genes) displaying SNP polymorphism could be easily developed. For example, Huang *et al.* (2004) showed that the R3 resistance to *P. infestans* in potato is conferred by two closely linked R genes named R3a and R3b. Biotests do not indicate which process leads to the bypassing of the R3 resistance locus. A *P. infestans* isolate is considered to have overcome the R3 locus when it can infect potato containing this R3 resistance locus, that is, when *P. infestans* isolates have both avr3a and avr3b virulent alleles. However, nothing is known about how mutations occured both in the Avr3a and in the Avr3b genes and how they were selected and maintained in populations. A better understanding of this could be achieved by developing molecular tools similar to the one detailed here and by artificially selecting virulent strains on a potato cultivar containing only the R3 resistance locus.

The early use of potato cultivar containing R3 resistance locus during the twentieth century have led to rapid apparition of strains able to bypass potato resistance (Turkensteen 1993). Although the R3 gene was not already used in current potato cultivars, virulence seemed to be largely maintained (90% of sampled strains in the study of Montarry *et al.* 2010b). The question of fitness cost associated with unnecessary virulence factors has thus been raised (Montarry *et al.* 2010b) and could be enriched by the use of our real-time quantitative PCR. Indeed, as in the pathosystem presented by Janzac *et al.* (2010), there was no measurable fitness cost via single infections for *P. infestans* but high cost of competitiveness between a virulent and an avirulent strains could be highlighted. The molecular tool developed here could be a good way to test such a cost in *P. infestans*, which could bring a better understanding of evolutionary consequences of virulence emergence. In a context of intense research on durable resistance, this kind of studies is on primarily importance to predict pathogen evolution with the use of resistance cultivar and then to assess their efficacy overtime.

In conclusion, our real-time quantitative PCR assay is the first diagnostic tool that allows the detection and quantification of an oomycete at the intraspecific level. It offers new opportunities to answer scientific questions from different fields from ecology to epidemiology and already revealed its potentialities in dealing with the problem of multiple infections.

Materials and methods

Selection of *Phytophthora infestans* reference isolates for Avr3a and avr3a

To develop the assay, six isolates of *P. infestans* (BEK, BP3, BP6, OEK, P46 and P8; Table S1) were chosen for showing different responses on the potato clone from the international late blight set containing the R3 (R3a + R3b) resistance locus: BP3 and OEK *P. infestans* isolates are avirulent (i.e. inducing a hypersensitive response of the host and no infection), and BEK, BP6, P46 and P8 *P. infestans* isolates are virulent (i.e. developing sporulating lesions). DNA from each *P. infestans* isolate was separately extracted from lyophilized mycelium cultured in liquid pea medium. These DNA extractions were performed using the DNeasy Blood & Tissue kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. The Avr3a gene was amplified by PCR as described by Armstrong *et al.* (2005) using the

Pex147F (5′-CCATGCGTCTGGCAATTATGCT-3′) and Pex147R (5′-CTGAAAACTAATATCCAGTGA-3′) primers. PCR products were sequenced using the Sanger technology (Genoscreen, Lille, France) in both directions. Chromatograms reading/correction and multiple alignments were carried out using the Chromas© software version 2.33 (Technelysium Pty Ltd, South Brisbane, Qld, Australia) and the BioEdit© sequence alignment editor version 7.0.9.0 (Hall 1999), respectively. Two independent PCR/sequencing procedures were performed for each isolate to produce error-free data sequence.

Cloning and creation of standards for real-time PCR assay

Cloning of Avr3a/avr3a genes was performed using the StrataClone™ PCR Cloning kit (Agilent Technologies – Stratagene Products, Massy, France) in the cloning vector pSC-A-amp/kan following the manufacturer's protocol. The experiment took place in three steps: PCR amplification (using primers Pex147F and Pex147R detailed above), ligation of PCR products in the pSC-A-amp/kan vector and bacterial transformation. Plasmid DNA was purified from recombinant bacteria using the Miniprep NucleoSpin plasmid extraction kit (Macherey-Nagel EURL, Hoerdt, France) and sequenced using appropriate T3 (5′-ATTAACCCTCACTAAAGG-3′) and M13-20 (5′-GTA AAACGACGGCCAGT-3′) universal primers that framed the site where Avr3a/avr3a sequences were theoretically inserted in the pSC-A-amp/kan plasmid. Fractions containing plasmid with BP3 (Avr3a) and BP6 (avr3a) sequence were chosen to constitute the Avr3a and avr3a standards, respectively. The number of plasmid copies in these fractions was assessed by quantifying DNA concentrations with a spectrophotometer (Nanodrop 2000, Thermo Scientific Inc., Wilmington, DE, USA). According to the concentration of plasmid, the fractions were 10-fold serially diluted to create series of fractions containing from 10^2 to 10^8 copies per μl. These serially diluted fractions were used as standards in the real-time PCR runs.

Molecular marker development

Using the Primer Express® software (Applied biosystem, Life Technologies SAS, Saint-Aubin, France), primers and TaqMan®-MGB probes were designed to perform real-time PCR assays. The sequence of the 'F' forward primer (5′-GCGAATCTAAATGAGGAGATGTTTAAT-3′) was selected to be specific to *P. infestans*. The 'R^{Avr3a}' reverse primer (5′-CCTGTTGTGCTGCCACCAA-3′) and the $probe^{Avr3a}$ (5′-FAM-CTGCTCTCTTCGTCAAC-MGB-3′) were designed according to the BP3 sequence, while the 'R^{avr3a}' reverse primer (5′-CCTGTTGTGCTGCCACCAC-3′) and the $probe^{avr3a}$ (5′-Vic-TGCTCTCTCCGTCAAC-MGB-3′) were designed to be specific to BP6 sequence (see Fig. 1 for complementary information).

Real-time PCR assay

The reactions were performed in a final volume of 25 μl using 12·5 μl of the TaqMan® Master Mix reagent kit (Qiagen). In addition to the 'F' primer, reactions were set up with Avr3a- or avr3a-specific reverse primer and appropriate probe, that is, $probe^{Avr3a}$ and $probe^{avr3a}$ for specific detection/quantification of Avr3a and avr3a, respectively. Each reaction required 0·8 μmol l^{-1} of forward and reverse primers, 0·2 μmol l^{-1} TaqMan®-MGB probe and 1 μl of DNA sample. PCR were performed using the Roche LightCycler 480 (Roche Diagnostics, Meylan, France). The amplification programme included an initial step at 95°C for 10 min followed by 45 cycles corresponding to (i) denaturation at 95°C for 15 s and (ii) hybridization and elongation at 60°C for 60 s. The fluorescent signal was acquired at the end of each hybridization/elongation step.

The 10-fold serially diluted fractions of Avr3a and avr3a plasmids (see Reliability in mixed DNA fractions) were used as quantification standards for the LightCycler 480 calibration curves specific to the Avr3a and avr3a quantification assays, respectively. To ensure a specific detection of each type of sequence, fractions containing Avr3a DNA and avr3a DNA were analysed using the F-R^{avr3a}–$Probe^{avr3a}$ and the F-R^{Avr3a}–$Probe^{Avr3a}$, respectively. Finally, series of artificial mixtures containing from 10^2 to 10^8 of each of the two types of DNA sequences (both Avr3a and avr3a DNA) were prepared and analysed using the different primers/probe combinations to test both the reliability and the specificity of each version of the real-time PCR assay (Avr3a- and avr3a-specific). This complete procedure was repeated four times.

Extending the reliability of the real-time PCR assay and counting zoospores

To extend the reliability of the real-time PCR assay to a genotyping procedure of the SNPs targeted in the Avr3a gene, six supplementary *P. infestans* isolates (P13, P43, PON05, PON10, PP1 and Z0; Table S1), with different geographical origin (Northern France or Western France) and collected between 2005 and 2008, were used as samples. DNA, extracted from lyophilized mycelium, was tested with the developed real-time PCR assays. Results were compared to the Avr3a gene sequences of these *P. infestans* isolates that were previously determined as described above [see Between-test variation (reproducibility)].

To give a biological dimension to the presented real-time PCR assay, DNA copy numbers were correlated to numbers of zoospores: the mononucleate infective spores of *P. infestans*. Suspensions of zoospores from six homozygous isolates (BEK, BP6, P46, PON05, PON10 and PP1), obtained from infected leaflets according to the procedure described by Clément *et al.* (2010), were counted eight times, each in 10 μl, with a microscope. Suspensions of zoospores (5 μl) were individually centrifuged during 10 min at 17 000 ***g***. DNA extractions were carried out using the resulting zoospores with the DNeasy Blood & Tissue kit (Qiagen) according to manufacturer's instructions. Quantification was performed using the appropriate real-time PCR assay to assess DNA copy number of each sample, and correlation between zoospore counts and DNA quantification were performed.

Data analysis

Fluorescence data were analysed with the LightCycler 480 software (v 1.5.0; Roche Diagnostics, Meylan, France) using the fit-point method. For each theoretical number of DNA copies present in the tested Avr3a and avr3a fractions, the means of the four replicated quantifications were calculated. This procedure was extended to mixed fractions containing Avr3a/avr3a ratios corresponding to 50/50, 10/90, 90/10, 1/99 and 99/1. Multiple mean comparisons between theoretical and estimated quantifications were performed using the pairwise t-test function of the R software version 2.11.1 (R Development Core Team 2005). Finally, the correlation between means of zoospore counting and the real-time PCR quantification associated with the corresponding extracted DNA fraction was tested using the correlative test function (cor.test).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Characteristics of *Phytophthora infestans* strains used for the development of the real-time PCR assay.